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Abstract:	Although proof–of-concept has been established showing that dissociated embryonic tooth germ cells can re-associate to form functional (bioengineered) teeth, the success of such re-associations relies on freshly isolated cells that have not been expanded in vitro. In addition in vitro expanded adult cell populations such can also contribute to bioengineered tooth formation but only as cells that respond to tooth inductive signals. Since the success of whole tooth bioengineering is predicated on the availability of large numbers of cells, in vitro cell expansion of tooth-inducing cell populations is an essential requirement for further development of this approach. We describe here a cell mixing approach that is used to evaluate the contributions of cell populations to bioengineered tooth formation. Using genetically-labelled cells we are able to identify the formation of tooth pulp cells and odontoblasts in bioengineered teeth. We show that although cultured embryonic dental mesenchyme cells are unable to induce tooth formation, they can contribute to tooth induction and formation if combined with non-cultured cells. Moreover we show that teeth can form from cell mixtures that include embryonic cells and populations of adult dental pulp cells but these cells are unable to contribute to formation of pulp cells or odontoblasts and at rations of 1:1 inhibit tooth formation. These results indicate that although in vitro cell expansion of embryonic tooth mesenchymal cells renders them unable to induce tooth formation, they do not lose their ability to contribute to tooth formation and differentiate into odontoblasts. Adult pulp cells however lose all tooth inducing and tooth forming capacity following in vitro expansion and at rations above 1:3 adult to embryonic cells, inhibit the ability of embryonic dental mesenchyme cells to induce tooth formation.

Mesenchymal cell community effect in whole tooth bioengineering

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Abstract

In vitro expanded cell populations can contribute to bioengineered tooth formation but only as cells that respond to tooth inductive signals. Since the success of whole tooth bioengineering is predicated on the availability of large numbers of cells, *in vitro* cell expansion of tooth-inducing cell populations is an essential requirement for further development of this approach. We set out to investigate if the failure of cultured mesenchyme cells to form bioengineered teeth might be rescued by the presence of uncultured cells and in order to test this we deployed a cell mixing approach that was used to evaluate the contributions of cell populations to bioengineered tooth formation. Using genetically-labelled cells we are able to identify the formation of tooth pulp cells and odontoblasts in bioengineered teeth. We show that although cultured embryonic dental mesenchyme cells are unable to induce tooth formation, they can contribute to tooth induction and formation if combined with non-cultured cells. Moreover we show that teeth can form from cell mixtures that include embryonic cells and populations of adult dental pulp cells but these cells are unable to contribute to formation of pulp cells or odontoblasts and at ratios of 1:1 inhibit tooth formation. These results indicate that although *in vitro* cell expansion of embryonic tooth mesenchymal cells renders them unable to induce tooth formation, they do not lose their ability to contribute to tooth formation and differentiate into odontoblasts. Post natal pulp cells however lose all tooth inducing and tooth forming capacity following *in vitro* expansion and at ratios above 1:3 postnatal to embryonic cells, inhibit the ability of embryonic dental mesenchyme cells to induce tooth formation.

Introduction

Whole tooth bioengineering has long been a goal of regenerative dentistry that despite some recent progress still has a number of difficult challenges to overcome. Tissue recombination experiments demonstrated sequential signalling between dental epithelium and mesenchyme (Mina and Kollar 1987; Lumsden 1988). This was followed by experiments that showed the ability of embryonic tooth germ cells to re-aggregate following dissociation and form teeth (Yamamoto et al 2003) and has since been used as a model to explore whole tooth bioengineering (Nakao et al. 2007, Ikeda et al. 2009, Nait Lechguer et al. 2008, Ohshima et al. 2011). Thus epithelium and mesenchyme tissues from E14.5 and E12.5 stage mouse tooth germs can be separated, the cells dissociated and recombined to form normal teeth. The reciprocal tissue induction that takes place during the early stages of tooth development whereby the epithelium first induces tooth formation in the mesenchyme and this is followed by a reciprocal induction for mesenchyme to epithelium, has been utilised to suggest a basis for whole tooth bioengineering that could utilise adult cells (Jernvall and Thesleff 2000, Tucker and Sharpe 2004, Zhang et al 2005). Thus when mesenchyme cells derived from adult bone marrow are combined with inductive stage embryonic dental epithelium, tooth formation is induced and the adult mesenchymal cells respond and fully contribute to tooth development (Ohazama et al. 2004). To date the only cells that have been shown to be capable of tooth inductive capacity are embryonic cells (epithelium or mesenchyme) isolated from tooth germs (Ohazama et al. 2004, Angelova Volponi et al. 2013). Furthermore in all experiments reported to date the inductive cells, whether epithelial or mesenchymal do not retain their inductive capacity following *in vitro* expansion (Zheng et al 2016). This thus poses a major problem in tooth bioengineering. Clearly the use of fresh (uncultured) embryonic tooth germ cells

is not feasible in any clinical context. Generation of cell lines from embryonic inductive cells is also not feasible since these cells lose their inductive capacity, in addition to any issues use of such allogeneic cells may have for generation of non-essential organs such as teeth.

Adult cell populations have been shown to be capable of forming bioengineered teeth as recipient cells combined with inductive embryonic cell populations (Duallibi et al. 2004, Takahashi et al. 2010). Thus as stated above, adult bone marrow mesenchymal cells can do this [Ohazama et al. 2004], as can adult gingival epithelial cells (Angelova Volponi et al. 2013). The challenge therefore is to identify adult cell populations that can be expanded in large numbers, ideally as autologous cells, where one population, either mesenchyme or epithelium has tooth inducing capacity.

The rapid loss of tooth inducing capacity by embryonic tooth germ cells when expanded *in vitro* is not understood and as a way of beginning to understand this process we investigated whether this ability might be rescued by embryonic cells. We found that uncultured embryonic tooth germ mesenchymal cells were able to rescue cultured cells and enable them to fully participate into bioengineered tooth development, forming pulp cells and odontoblasts. However, this rescue effect was not observed with postnatal dental pulp mesenchyme cells, despite these being of the same developmental origin as the embryonic cells. The rescue of cultured cells by the presence of fresh cells shows characteristics of the community effect identified during embryonic development as a process that enables mixtures of different cells to differentiate along the same pathway.

Materials and Methods

Experimental mouse strains

In order to trace the origins of the cells contributing to the tooth primordia formation in epithelium-mesenchyme recombination experiments, three different strains of mice were used, respectively, wild-type mice (CD-1 mice), transgenic mice expressing green fluorescent protein (GFP mice) and transgenic mice expressing membrane-targeted red fluorescent protein (tandem dimer Tomato, tdTomato) prior to Cre recombinase exposure (mTmG mice). All animal procedures conformed to the ARRIVE guidelines and in according with UK Home Office regulations.

Isolation of embryonic dental epithelium and mesenchymal cells

Intact bilateral molar tooth germs were dissected from E12.5 (for epithelial tissue) and E14.5 (for mesenchymal embryonic cells) mouse embryos, utilizing sterile fine needles in Leibovitz's L-15 medium (L-15, Gibco®, 21083-027). After being trimmed from the surrounding tissue, tooth germs were transferred into 1.2 U/ml Dispase and incubated at 37°C for 40 minutes. After being washed in L-15 medium, epithelium and mesenchyme of the tooth germs were mechanically separated with fine needles. The embryonic mesenchymal cells were obtained by digesting the isolated mesenchymal tissue with Trypsin (TrypLE™ Express) and used as fresh (dissociated cells) from wildtype (CD1) mice, or cultured from GFP (green) mice and recombined with epithelial tissue (mTmG mice). The embryonic dental mesenchymal cells were re-suspended in complete alpha-minimal essential medium (α-MEM, BioWhittaker® BE02-002F) with 15% fetal bovine serum (FBS, Gibco® 10270-106), 1% antibiotic-antimycotic (Gibco® 15240-062) and 0.1mM L-ascorbic acid (Sigma-Aldrich 49752), plated in 6-well cell culture plates or 75cm^2 cell culture flasks (CELLSTAR®,) and then incubated at 37°C, 5% carbon dioxide (CO_2) for 7 days. The embryonic mesenchymal cells were used at P0 (passage 0).

Postnatal dental pulp cells were established from molars of GFP (green) mice pups (7 days old). Postnatal molar pulp pieces were digested in mixture of enzyme containing 2mg/ml Collagenase D (Roche) and 120 unit/ml Dispase (Roche) in PBS for 50 minutes, blocked with complete α-MEM and filtered through 70µm cell strainer (BD Falcon™ 352350) to obtain a uniform single cell suspension. These cells were cultured in 6- well plate at density of 1.2-1.5×10^5 cells/ well. Medium was changed every 2-3 days, and passaging was performed when the cells were 70% confluent. These cells were used in recombination experiments with E12.5 embryonic tooth germ epithelium at P1 (passage 1).

Recombination of epithelium and mesenchymal cells

Recombinations were carried out previously described (Angelova-Volponi et al 2013). Briefly, mesenchymal cells (2×10^5) were centrifuged in a PCR tube (0.2 ml, STARLAB) to form a pellet and then injected on the top of 4-5 pieces isolated E12.5 uncultured epithelium tissue using sharpened fine pipette tips (20µl, GELoader®) in a 25 µl gel drop of Cellmatrix type I-A (Nitta gelatin, Osaka, Japan), placed on a cell culture insert (4.0-µm pore size; BD, Franklin Lakes, NJ, USA). The re-combination was cultured for 9-11 days on the cell culture insert containing 1.5 mL/well complete αMEM.

Renal transfers

The surgical transfer of tissue explants (recombinations) into renal capsules was performed as previously described (Angelova-Volponi 2013). Samples were scanned using a Skyscan 1272 Bruker micro CT and analysed with Microview software (GE).

Imaging

Recombination samples were fixed in 4% PFA for 20 minutes, washed 3 times for 5 minutes in in 1x PBS and mounted on slides utilizing mounting medium with DAPI (VECTASHIELD® H-1200). Coverslips on top of the specimen were sealed around the perimeter with nail polish. Fluorescent tomography scans were performed on processed samples using z-stack imaging programme on a confocal microscope (Leica TCS SP5).

Results

Cell mixing of embryonic tooth germ cells

In order to develop a foolproof experimental protocol that could be used to test the contribution of mesenchymal cell populations to bioengineered tooth formation we utilised genetically-marked mouse lines to follow cell fates. Since mesenchymal cell contamination is commonly observed following physical separation of embryonic dental epithelium from developing tooth germs, we used isolated epithelium at E12.5 embryonic stage, from a mouse reporter (mTmG mice) line where all cells are marked red, with fresh (dissociated) cells of wildtype (CD1 mice -no fluorescent expression) and cultured embryonic mesenchymal cells from transgenic mice expressing green fluorescent protein (GFP mice) (Figure 1). In this way, any contamination with mesenchymal cells could be easily detected. We used a GFP reporter line for the isolation of mesenchymal cells whose fates was to be followed during bioengineered tooth formation and combined these with wildtype, uncultured mesenchymal cells.

We first verified the methodology by recombining fresh, dissociated (uncultured) E14.5 dental mesenchymal cells with E12.5 fresh epithelial tissue. In this recombination, the epithelial cells have not yet received inductive cells and thus the mesenchymal cells are expected to induce tooth formation in the epithelial tissue. As expected tooth germs were observed in 100% of the recombination experiments (Table S1). In order to determine if cultured embryonic E14.5 tooth mesenchyme cells could induce tooth formation we combined GFP positive (green) cells with E12.5 tooth germ epithelium and in these combinations no tooth germs were observed (n=11) (Table S1 and Fig.S2B).

We next set out to investigate if the presence of uncultured E14.5 mesenchyme cells with cultured cells was sufficient to enable the cultured cells to participate in tooth formation. E14.5 cultured mesenchyme cells were mixed with fresh (dissociated) wildtype cells of E14.5 embryos in different ratios (Table S1). The mixtures of cells were recombined with embryonic epithelial tissue at stage E12.5 (mTmG mice) line where all cells are marked red. After 11 days in culture, we observed tooth germ formation in the experiments where the cultured embryonic mesenchymal cells constituted 10%, 50% and 75% of the mesenchymal cell mixture (Table S1; Figure 2), with clear participation of the cultured cells to the tooth pulp (Figure 2 A3,4,,B3,4,C3,4). Cultured cells were also clearly visible (GFP positive, green) adjacent to epithelium, exhibiting an elongated appearance characteristic of odontoblasts (Figure 2 A4, B4 and C4). When the embryonic mesenchymal cultured cells constituted 90% of the cells used in the mixture with 10% uncultured (fresh) cells, no formation of tooth germs was observed (n=10) (Table S1 and Figure 4 A,B). Although there was variation in the numbers of GFP^+ cells observed in the tooth germs within a single experiment, there was a consistent general trend of increased cultured cell contribution with increased cell number. Significant variation in tooth germ shape was also observed in all recombinations regardless of the ratio of fresh to cultured cells. This is a usual phenomenon we observe with this form of recombinations and is illustrated in Fig. S3.

To study if the mixtures of cultured and fresh tooth cells could develop further to minerisation stages and initiate root development, subcapsular transplantations of tooth-like structures/tooth primordia in mouse kidney were performed. After 9 days of *in vitro* culture, tooth-like structures/tooth primordia formed in recombinations using cell mixtures consisting of 75% cultured GFP and 25% fresh CD1 E14.5 molar germ mesenchymal cells and mTmG E12.5 molar germ epithelial tissue were transplanted into kidney capsules of adult (4-6 weeks) CD-1 mouse hosts. The host mice were sacrificed after 4 wks, and the implants were separated from the kidney

capsules and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. After being washed with PBS, specimens for microCT were scanned by Skyscan 1272 Bruker microCT scanner. The specimens were scanned to produce 6.5-µm-voxel-size volumes, with an x-ray tube voltage of 80 kVp and a tube current of 80 µA. An aluminum filter (0.05 mm) was used to adjust the energy distribution of the x-ray source. The specimens were characterized further by three-dimensional slice volumes, generated and measured with Microview software (GE). After microCT scan, specimens were embedded and mounted on slides for confocal fluorescent tomography scans as the procedure described in "Imaging" for recombination samples.

Cell mixing of postnatal dental pulp cells

Postnatal dental pulp cells were isolated from molars of 7 day old transgenic mice expressing green fluorescent protein (GFP) and expanded in culture for 10 days (passaged once; P1), prior to mixing with embryonic mesenchymal dissociated cells and recombined with epithelium (E12.5), of mTmG mice. Mixtures of 25%, 50% and 100% of cultured postnatal dental pulp cells with non-labelled (wildtype CD1 mice) dissociated embryonic mesenchymal cells, (Table 1) were combined with E12.5 epithelium. Tooth germ formation was observed after 11 days of culture, in the mixtures of 25% of cultured dental pulp cells (Figure 2). Although a few individual cultured dental pulp cells could be seen (Figure 3B, C, D) in 25% mixtures, they did not contribute to tooth germ formation, the majority of postnatal cells were excluded from the teeth formed in recombinations (Fig. 3A2). No tooth germ formation was observed in the mixture containing 50% or more cultured cells (Fig. 4).

These results suggested that the presence of postnatal mesenchyme cells is inhibiting the ability of embryonic cells to induce tooth formation. An alternative explanation is that since the postnatal

cells are non-inductive, the embryonic cells are the only inductive cells in the mixture and their number may be below the threshold for induction. We thus repeated the 50% cultured postnatal cell mixture with 4 times the number of cells (4 x 10^5 embryonic and 4 x 10^5 postnatal cells). Since we know that the 75% cultured embryonic cell experiments (0.5 x 10^5 fresh and 1.5 x 10^5 cultured embryonic cells) can induce tooth formation (Fig. 2C), 4 x 10^5 fresh embryonic cells should be sufficient for induction. No tooth germ formation was observed with these increased cell numbers suggesting that the postnatal cells are indeed inhibiting the tooth inducing capacity of embryonic cells.

Discussion

The ability to reproduce embryonic tooth formation from combinations of dissociated cells *in vitro* forms a general concept for bioengineered tooth formation. In all such cell combinations, one of the cell populations, either epithelium or mesenchyme, needs to have tooth inducing capacity. Thus for example, embryonic tooth epithelium isolated from an embryonic stage where it is inductive, can induce non-dental cells such as bone marrow stromal cells to form teeth (Ohazama et al 2004). Similarly, embryonic tooth mesenchymal cells from an inductive embryonic stage can induce formation in non-dental epithelium from adult gingiva (Angelova Volponi et al 2013). Since cells isolated from mid-gestation embryos are not usable in a clinical context, alternative inductive cell populations are required that can ideally be isolated from adult tissues and expanded *in vitro* to provide sufficient cell numbers. However even embryonic tooth inducing cells rapidly lose the inductive capacity following expansion *in vitro* and until the basis for this loss is understood, further, realistic progress towards a clinically-usable bioengineered tooth system is not possible.

The use of recombination of dissociated cells as an assay for cell inductive capacity in organ formation relies on the ability to obtain populations and epithelial and mesenchymal cells that are

not cross-contaminated. Since cells proliferate in *ex-vivo* tissue recombinations/reassociations, contamination of one population with even a small number of cells of the other population could lead to misleading results. We validated our cell dissociation methodology by using genetically-labelled cell populations that can be easily distinguished by fluorescent markers.

The formation of tooth primordia from dissociated embryonic tooth epithelial and mesenchymal cells has been reported to require a minimum of 13.5 $\pm$0.5 x 10^4 mesenchymal cells when recombined with epithelial cells (Nait Lechguer et al 2008). We have also observed a minimum cell number requirement of 15.2 $\pm$0.5 x 10^4 mesenchymal cells in our recombination experiments. In our cell mixing experiments with cultured embryonic mesenchyme cells, we used a total mesenchyme cell number of 2.0 x 10^5. Tooth formation was observed with inductive mesenchyme cell numbers ranging from 5x10^4 to 1.8x10^5 in the mixture. Thus in the mixture containing 5x10^4 (25%) inductive mesenchyme cells with 1.5 x10^5 (75%) cultured there are insufficient inductive cell alone for tooth formation. This suggests that the cultured cells are likely to be actively participating in tooth induction.

If current methods are to be used in any therapeutic context to generate bioengineered human teeth, the cell numbers needed will have to be obtained from *in vitro* cell expansion. However, it has been established that culture of inductive embryonic tooth primordia cells results in a rapid loss of inductive capacity (Zheng et al 2016). *In vitro* cell expansion does not affect the ability of either epithelial or mesenchymal cells to participate in tooth formation, and thus obtaining sufficient cell numbers of a recipient cell population does not present a problem for bioengineering (Angelova Volponi et al. 2013, Ohazama et al. 2004).

During embryonic development a process called the “community effect” can act to allow heterogeneous cell populations to differentiate down a common pathway by cells interacting with

their immediate neighbours (Gurdon et al 1993). In order to begin to understand the cellular basis for this rapid loss in inductive capacity in cell culture we set out to investigate if an artificially generated community effect whereby inductive tooth mesenchymal cells (namely uncultured embryonic cells), might rescue the loss inductive capacity following expansion *in vitro*. We showed that as few as 25% inductive tooth mesenchyme cells when mixed with non-inductive tooth mesenchyme cells were sufficient to induce tooth formation and for the non-inductive cells to fully participate in tooth formation, including differentiation into odontoblasts. At proportions of 90% and above of non-inductive cells, no tooth formation was observed. This observation is consistent with mathematical models of the community effect that show that cell density must be above a critical threshold for the effect to occur (Saka et al 2011). The embryonic community effect is most often described in terms of cell signalling regulating transcription to generate cell homogeneity (Gurdon et al 1993). Implicit in this is the assumption that all cells in the community express common signalling receptors and thus we assume that the most likely cause of the loss in tooth inductive capacity is loss of cell signal secretion rather than loss of receptors.

Dental pulp cells of adult teeth can be easily cultured *in vitro* as heterogeneous populations that contain cells with stem cell-like properties (Gronthos et al. 2000, Gronthos et al. 2002, Jo et al. 2007, d'Aquino et al. 2007, Huang et al. 2009, Koyama et al. 2009, Waddington et al. 2009, Balic et al. 2010, Angelova Volponi and Sharpe 2013). These cells have the same embryonic origin (cranial neural crest) as tooth primordia mesenchyme cells and are thus could candidates for cells that might be used in tooth bioengineering. We thus investigated whether postnatal tooth pulp cells are able to participate in the community effect provided by embryonic inductive cells. Proportions of postnatal pulp cells above 25% in mixtures with inductive mesenchyme cells failed to form teeth. At 25% and below although teeth formed there was no contribution of postnatal cells to tooth formation. Tooth formation failure occurred with an inductive cell number of 1.0×10^5

(50%) that in the absence of any postnatal cells in the mixture is sufficient to induce tooth formation. We increased this number to 4.0 x 10^5 cells and still failed to observe any tooth formation when mixed with an equal number of postnatal pulp cells. The presence of adult tooth pulp cells thus appears to inhibit the ability of embryonic cells to induce tooth formation. Although this could be a simple dilution effect we believe this is unlikely since even with increased numbers of inductive cells there was still no evidence of tooth formation. Quite why postnatal pulp cells should have this effect is not currently understood. Adult mesenchyme cells are able to fully participate in bioengineered tooth formation as recipient cells for epithelial inductive signals and so the inhibition of tooth formation we observe in the mixtures is most likely prevention of inductive signals from the embryonic mesenchyme. One possible mechanism may be the secretion of signalling inhibitors by the postnatal pulp cells. Understanding the molecular basis of this phenomenon is important for future progress in tooth bioengineering.

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Figure legends

Figure 1. Schematic representation of recombinations using cell mixtures. (A) Cell culture: *in vitro* expansion of GFP mouse E14.5 molar tooth germ mesenchymal cells or PN7 molar pulp cells; (B) Cell mixing: cultured GFP cells (either E14.5 mesenchymal cells or PN7 pulp cells) were mixed with non-cultured (fresh) CD1 E14.5 molar tooth germ mesenchymal cells at different ratios (cultured:fresh =1:9, 1:3, 1:1, 3:1, 9:1); (C) Recombination: mixtures of cultured GFP cells and non-cultured CD1 cells recombined with mTmG mouse E12.5 molar tooth germ epithelial tissue *in vitro* for 9-11 days. (* mes: mesenchyme; epi: epithelium)

Figure 2. Recombinations of mTmG E12.5 molar germ epithelial tissue (red) and mixtures of cultured GFP molar tooth germ mesenchymal cells (green) and CD1-wildtype (non-labeled) tooth germ dissociated cells.

Tooth-like structures formed in recombinations using cell mixtures consisting of no more than 75% cultured E14.5 tooth germ mesenchymal cells (A1, B1, C1 bright field).

A: 10% cultured GFP (green) and 90% fresh CD1 E14.5 molar germ mesenchymal cells; B: 50% cultured GFP and 50% fresh CD1 E14.5 l molar germ mesenchymal cells; C: 75% cultured GFP and 25% fresh CD1 E14.5 molar germ mesenchymal cells. Green (GFP+) cells could be seen adjacent to the epithelium (A2, B2, C2), exhibiting elongated appearance (A3, B3, C3) (Scale bar: 250μm in A1-2, B1-2, C1-2; 100μm in A3, B3, C3).

Figure 3. Tooth-like structures formed in recombinations using cell mixtures consisting of 25% cultured GFP positive postnatal (PN7) mouse pulp cells.

In recombinations of mTmG E12.5 molar germ epithelial tissue (red) and 25% cultured GFP PN7 molar pulp cells (green) and 75% fresh wildtype (CD1) E14.5 molar germ mesenchymal cells (non-labeled), tooth-like structures were formed after 11 days *in vitro* (A1). Few cultured GFP positive (green) cells could be seen inside the tooth primordia (B, C, D). Majority of adult cells (green) were excluded from the tooth-like structures (A2). (Scale bar: 250μm in A1-2, B, C; 100μm in D)

Figure 4. Failure of tooth formation in recombinations using cell mixtures consisting of 90% cultured E14.5 tooth germ mesenchymal cells or 50% cultured PN7 pulp cells. (A) Cyst-like structures rather than teeth were formed in recombinations of mTmG E12.5 molar germ epithelial tissue (red) and 90% cultured GFP E14.5 molar germ mesenchymal cells (green) and 10% fresh CD1 E14.5 molar germ mesenchymal cells (non-labeled), shown in bright field (A1) and confocal scan (A2).

B) 50% cultured GFP PN7 molar pulp cells (green) and 50% fresh CD1 E14.5 lower molar germ mesenchymal cells (non labeled), failed to form tooth-like structures, shown in bright field (B1) and confocal scan (B2). (Scale bar: 250μm).

Supplementary figure legends:

Table S1: Tooth formation success rate of mixed cell recombinations.

Figure S1. Schematic representation of positive and negative controls for mixed cell recombinations. (A) Positive controls: mTmG E12.5 molar tooth germ epithelial tissue recombined

with 100% non-cultured CD1 E14.5 lower molar tooth germ mesenchymal cells; (B) Embryonic negative controls: mTmG E12.5 molar tooth germ epithelial tissue recombined with 100% cultured GFP E14.5 molar tooth germ mesenchymal cells; (C) Postnatal negative controls: mTmG E12.5 molar tooth germ epithelial tissue recombined with 100% cultured GFP PN7 molar pulp cells. *epi: epithelium; mes: mesenchyme.

Figure S2. Positive and negative controls for mixed cell recombinations.

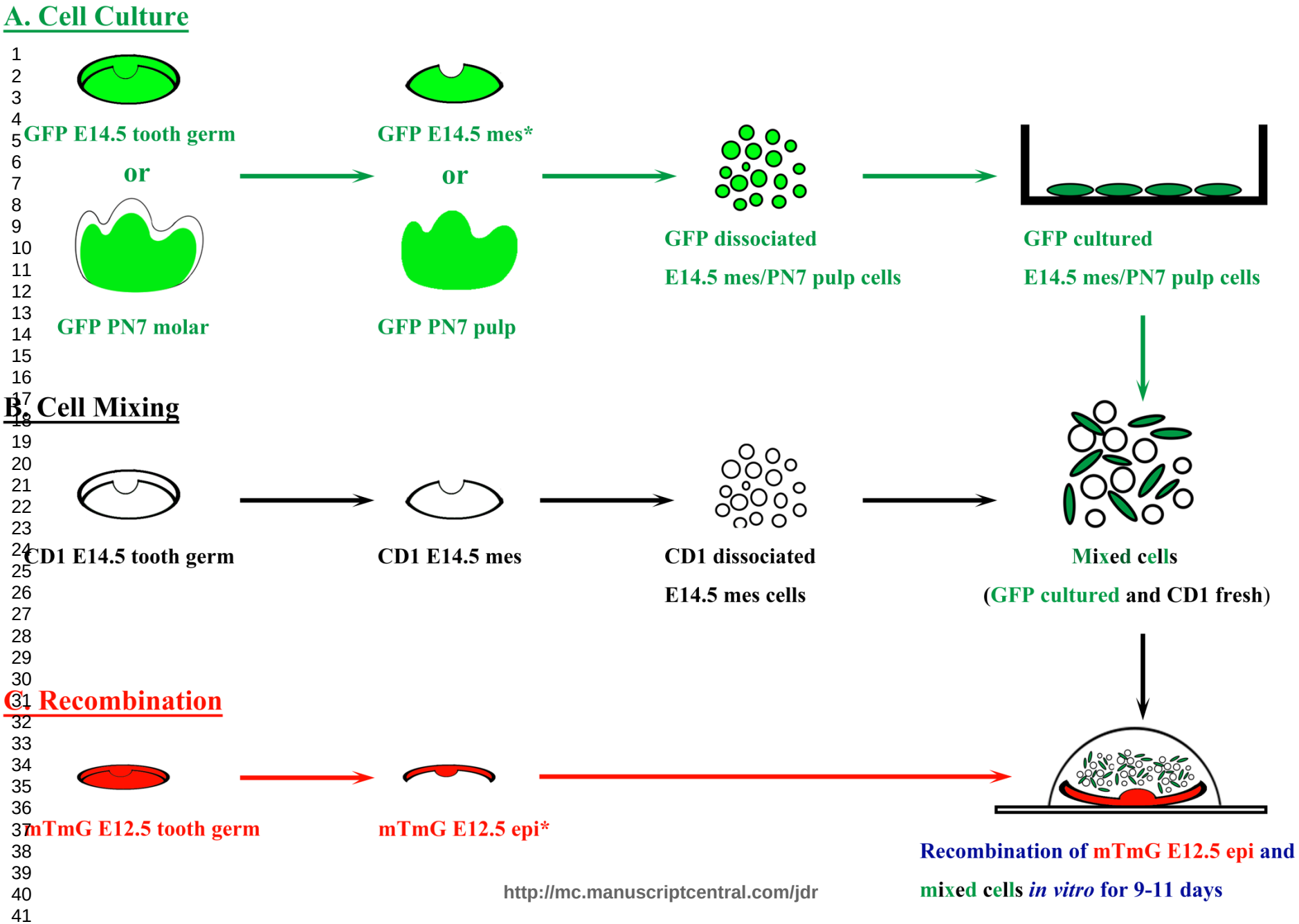
mTmG E12.5 tooth germ epithelial tissue (red) was recombined and cultured with: (A) non-cultured CD1 E14.5 tooth germ mesenchymal cells (non-labeled) where tooth-like structures formed (Positive control); (B) cultured GFP E14.5 molar germ mesenchymal cells (green) where cyst-like structures, "rolled-up" epithelium (red) surrounded by cultured mesenchymal cells (green), formed (Embryonic negative control); (C): cultured GFP PN7 molar pulp cells (green) where no tooth formation was observed (Postnatal negative control). (Scale bar: 250μm).

Figure S3. Tooth-like structures formed in one recombination using cell mixtures consisting of 50% cultured GFP positive E14.5 molar tooth germ mesenchymal cells. Different tooth shapes can be observed in the same recombination, including multiple distinct cusps (Fig. S3B), dual less distinct cusps (Fig. S3C) and single distinct cusp (Fig. S3D). Distribution patterns of GFP+ cultured cells in pulp are in general similar but slightly different from tooth to tooth. (Scale bar: 250μm in A1-2, B1, C, D; 100μm in B2).

Figure S4. After 9 days of *in vitro* culture, tooth-like structures/tooth primordia formed in recombinations using cell mixtures consisting of 75% cultured GFP and 25% fresh CD1 E14.5 molar germ mesenchymal cells and mTmG E12.5 molar germ epithelial tissue (A1, A2) were

transplanted under mouse renal capsules. Lumps of bone-like structures with embedded teeth were found after 4 weeks of *in vivo* culture (B). microCT scan (D1-2) and 3D reconstruction (C1-2) show that these teeth have calcified crowns (enamel), less calcified inside layer (dentine) and developing roots. (* Enm: Enamel; Dt: Dentine) (Scale bar: 250 μm)

Figure S5. A single tooth was isolated from implant lumps which originate from recombinations using cell mixtures consisting of 75% cultured GFP and 25% fresh CD1 E14.5 molar germ mesenchymal cells and mTmG E12.5 molar germ epithelial tissue (A1: bright field; A2: black field. Red dot lines outline the pulp cavity). Fluorescent view (A3) and confocal scan (B) show that GFP+ cells (cultured cells) evenly distributed in tooth pulp, both in the chamber and along the developing root. Higher magnification confocal scans (C1-2) show that GFP+ cells at the edge of pulp appear to have elongated segments as odontoblast processes (white arrows). (Scale bar: 250μm in A1-3, B; 100μm in C1-2)


A. Cell Culture
GFP E14.5 tooth germ
or
GFP PN7 molar
GFP E14.5 mes*
or
GFP PN7 pulp
GFP dissociated
E14.5 mes/PN7 pulp cells
GFP cultured
E14.5 mes/PN7 pulp cells
B. Cell Mixing
CD1 E14.5 tooth germ
CD1 E14.5 mes
CD1 dissociated
E14.5 mes cells
Mixed cells
(GFP cultured and CD1 fresh)
C. Recombination
mTmG E12.5 tooth germ
mTmG E12.5 epi*
Recombination of mTmG E12.5 epi and
mixed cells in vitro for 9-11 days

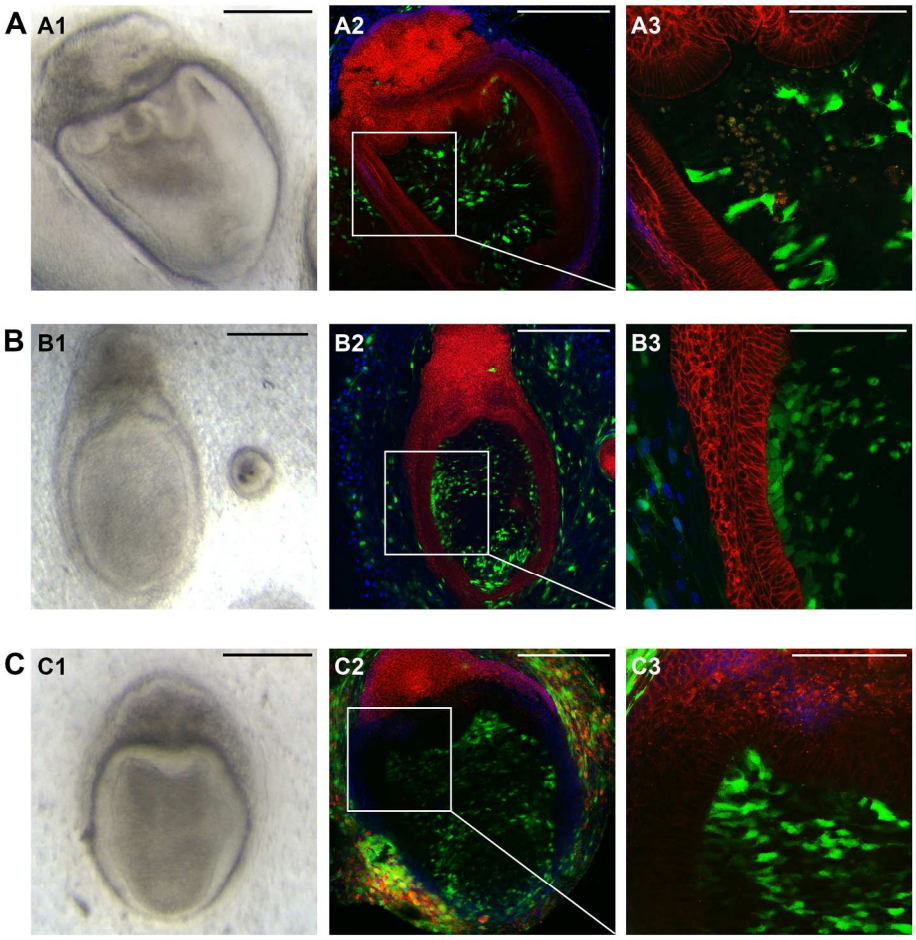


Figure 2. Recombinations of mTmG E12.5 molar germ epithelial tissue (red) and mixtures of cultured GFP molar tooth germ mesenchymal cells (green) and CD1-wildtype (non-labeled) tooth germ dissociated cells. Tooth-like structures formed in recombinations using cell mixtures consisting of no more than 75% cultured E14.5 tooth germ mesenchymal cells (A1, B1, C1 bright field).
A: 10% cultured GFP (green) and 90% fresh CD1 E14.5 molar germ mesenchymal cells; B: 50% cultured GFP and 50% fresh CD1 E14.5 l molar germ mesenchymal cells; C: 75% cultured GFP and 25% fresh CD1 E14.5 molar germ mesenchymal cells. Green (GFP+) cells could be seen adjacent to the epithelium (A2, B2, C2), exhibiting elongated appearance (A3, B3, C3) (Scale bar: 250μm in A1-2, B1-2, C1-2; 100μm in A3, B3, C3).

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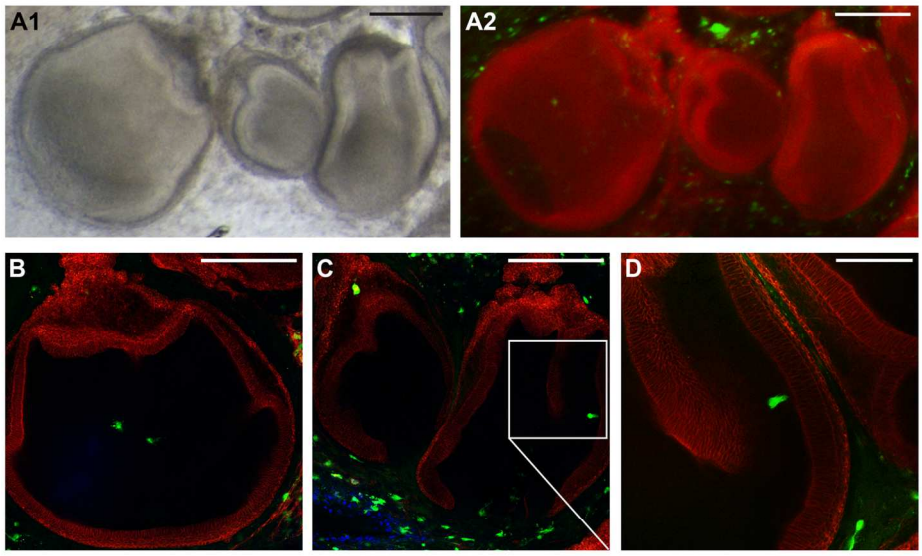

Figure 3. Tooth-like structures formed in recombinations using cell mixtures consisting of 25% cultured GFP positive postnatal (PN7) mouse pulp cells.
In recombinations of mTmG E12.5 molar germ epithelial tissue (red) and 25% cultured GFP PN7 molar pulp cells (green) and 75% fresh wildtype (CD1) E14.5 molar germ mesenchymal cells (non-labeled), tooth-like structures were formed after 11 days in vitro (A1). Few cultured GFP positive (green) cells could be seen inside the tooth primordia (B, C, D). Majority of adult cells (green) were excluded from the tooth-like structures (A2). (Scale bar: 250μm in A1-2, B, C; 100μm in D)

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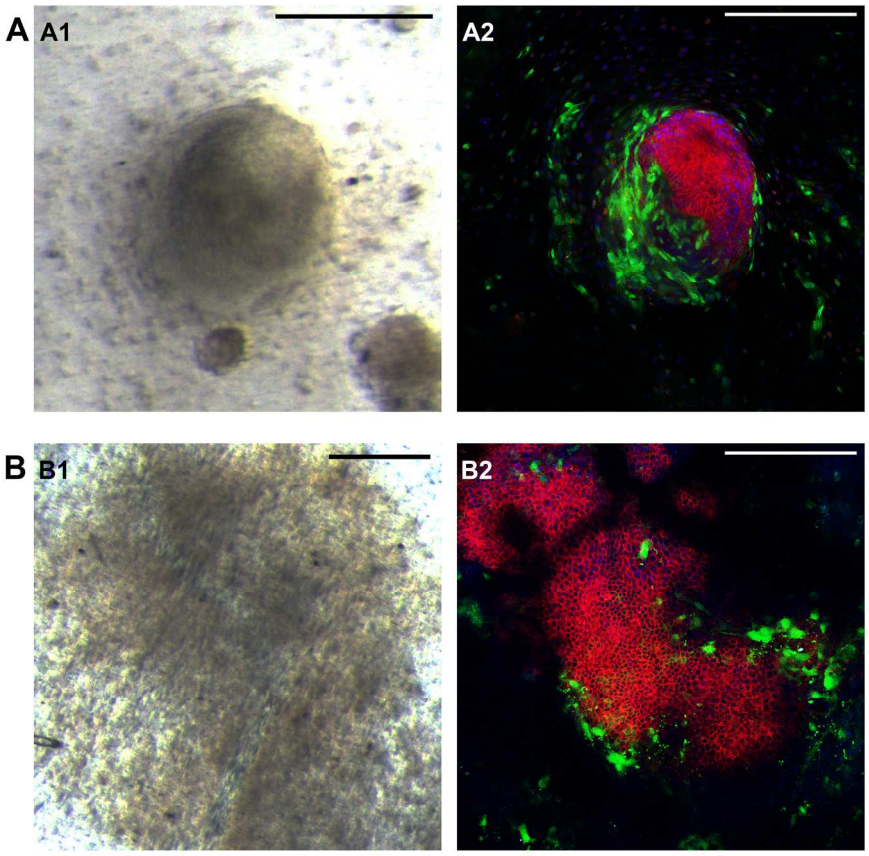

Figure 4. Failure of tooth formation in recombinations using cell mixtures consisting of 90% cultured E14.5 tooth germ mesenchymal cells or 50% cultured PN7 pulp cells. (A) Cyst-like structures rather than teeth were formed in recombinations of mTmG E12.5 molar germ epithelial tissue (red) and 90% cultured GFP E14.5 molar germ mesenchymal cells (green) and 10% fresh CD1 E14.5 molar germ mesenchymal cells (non-labeled), shown in bright field (A1) and confocal scan (A2).
B) 50% cultured GFP PN7 molar pulp cells (green) and 50% fresh CD1 E14.5 lower molar germ mesenchymal cells (non labeled), failed to form tooth-like structures, shown in bright field (B1) and confocal scan (B2). (Scale bar: 250μm).

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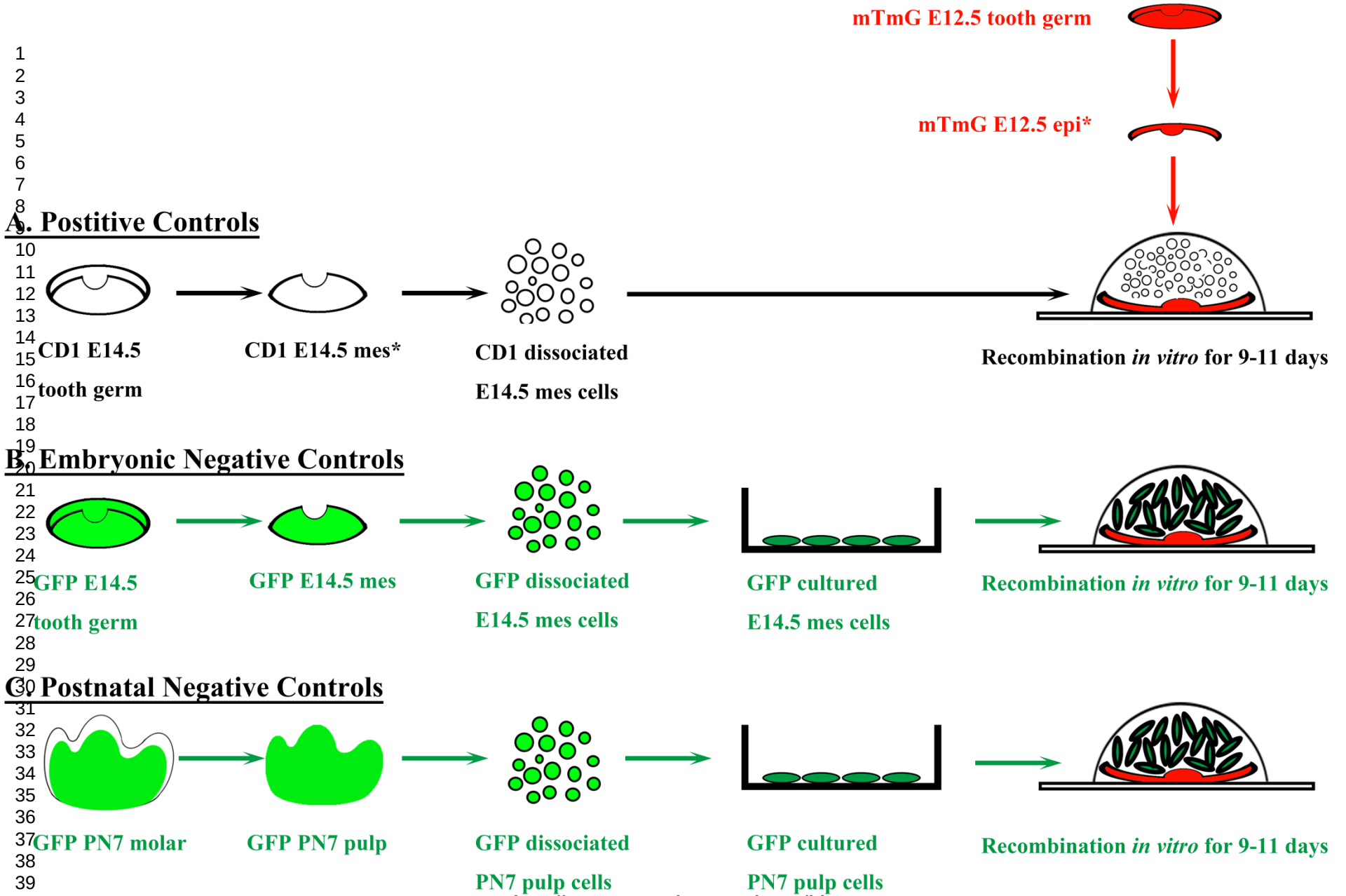
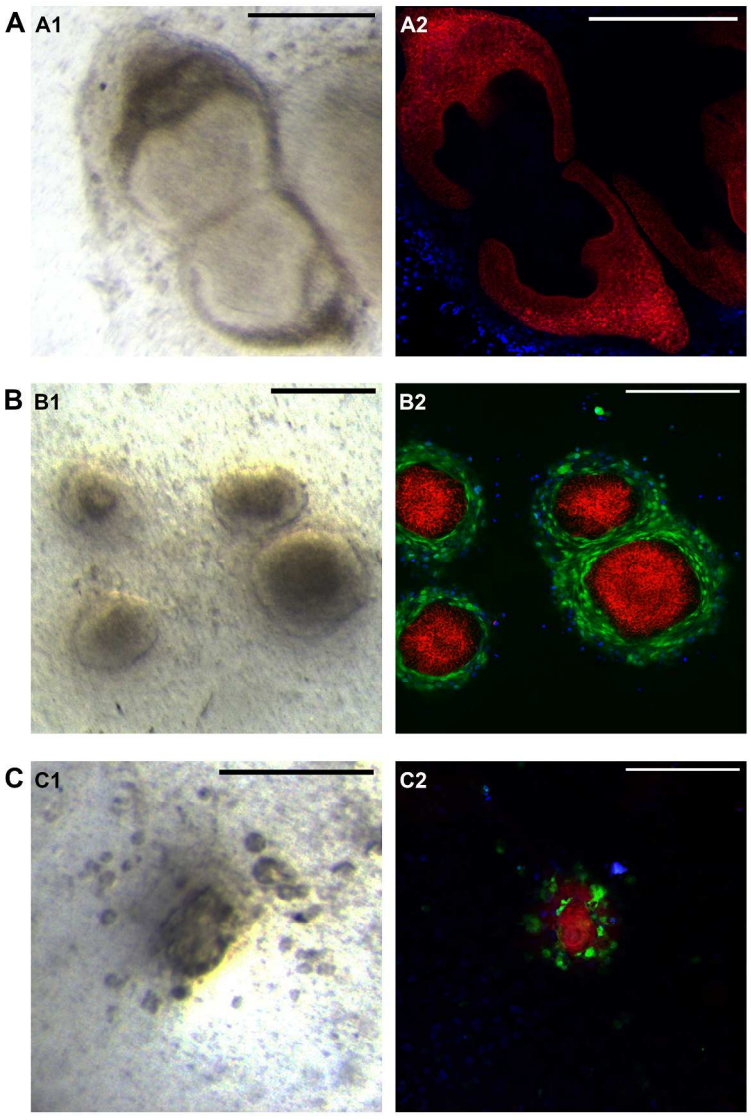

mTmG E12.5 tooth germ
mTmG E12.5 epi*
A. Postitive Controls
CD1 E14.5 tooth germ
CD1 E14.5 mes*
CD1 dissociated E14.5 mes cells
Recombination in vitro for 9-11 days
B. Embryonic Negative Controls
GFP E14.5 tooth germ
GFP E14.5 mes
GFP dissociated E14.5 mes cells
GFP cultured E14.5 mes cells
Recombination in vitro for 9-11 days
C. Postnatal Negative Controls
GFP PN7 molar
GFP PN7 pulp
GFP dissociated PN7 pulp cells
GFP cultured PN7 pulp cells
Recombination in vitro for 9-11 days



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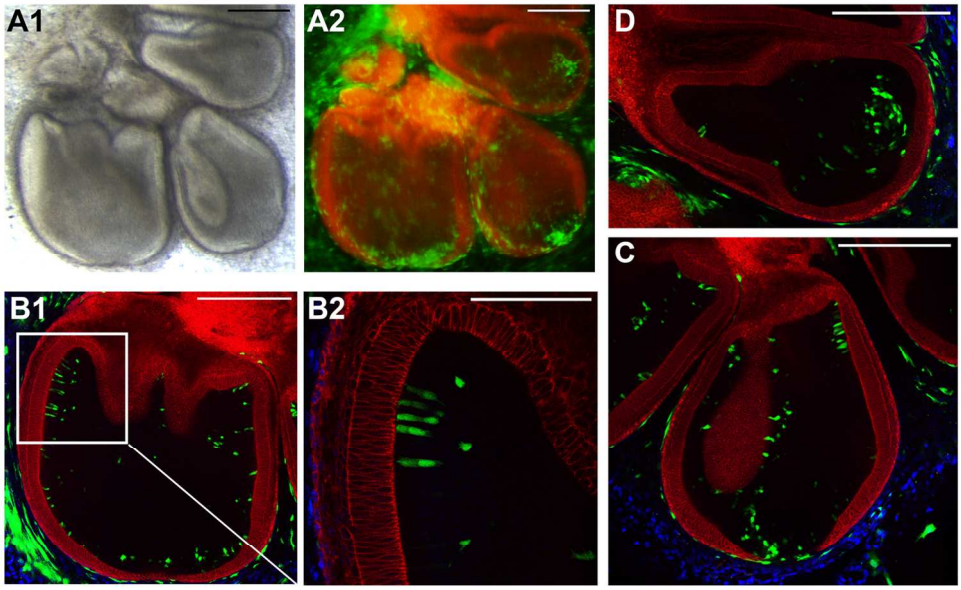

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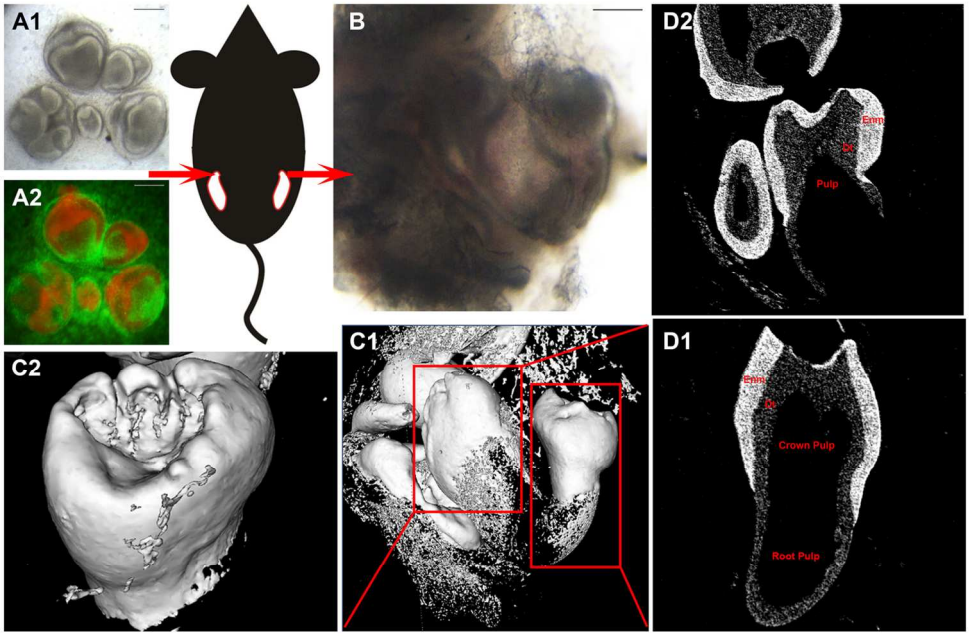

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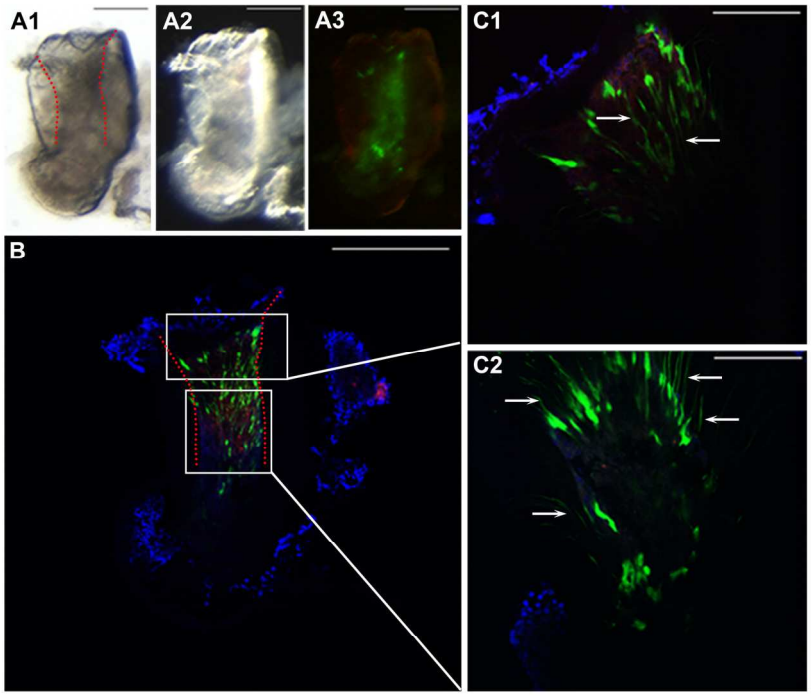

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ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	x
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	x
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	x
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	x
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	x
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	x
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	x
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	x

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	x
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	x
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	x
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	x
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	x
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	x
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	x
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	x
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	x
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	x
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	x
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	x

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.